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**13. ABSTRACT** *(Maximum 200 Words)*

Using differential cDNA sequencing approach, we first identified a breast cancer specific gene, **BCSG1**, which was expressed abundantly in metastatic breast cancer cDNA library but scarcely in normal breast cDNA library. Interestingly, BCSG1 revealed no homology to any other known growth factors or oncogenes; rather, BCSG1 revealed extensive sequence homology to neurotic proteins of α synuclein and β synuclein, and thus was also named as γ Synuclein (SNCG). Synucleins are emerging as a central player in the fundamental neural processes and in the formation of pathologically insoluble deposits characteristic of Alzheimer's (AD) and Parkinson's (PD) diseases. However, synucleins particular SNCG have also been implicated in non-neural diseases particularly in the hormone-responsive cancers of breast and ovary. SNCG expression is highly associated with breast cancer and ovarian cancer progression. In addition, overexpression of SNCG in breast cancer cells significantly stimulated cell growth in vitro and tumor metastasis *in vivo*. However, the molecular targets of SNCG aberrant expression for breast cancer have not been identified. For the first time, we report a chaperone-like activity of SNCG in stimulating the transcriptional activity of estrogen receptor-α (ER-α) in MCF-7 cells. Consistent with the stimulation of ER-α, SNCG stimulated the ligand-dependent cell proliferation. Demonstration of the stimulation of ER-α signaling as one of the cellular functions of SNCG will have a great impact on the biology of steroid receptors and the pathological role of SNCG on hormone-responsive tumors including breast, ovary, and prostate.

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## I. BACKGROUND AND SIGNIFICANCE

### I-1. Identification and cloning.

*Identification of BCSG1/SNCG.*

We undertook a search, using the differential cDNA sequencing approach as we previously described (1-3), for isolation of differentially expressed genes in the cDNA libraries from normal breast and breast carcinoma. Of many putative differentially expressed genes, a breast cancer specific gene, BCSG1, which was (a) highly expressed in mammary gland relative to other organs and was (b) high abundance in a breast cancer cDNA library but scarcely in a normal breast cDNA library, was identified as a putative breast cancer marker (1). The BCSG1 gene is transcribed into a 1 kb mRNA, and encodes a 127-amino acid polypeptide. Comparison of the predicted amino acid sequence with genetic databases reveals that BCSG1 is highly homologous to a family of neuronal cytosolic proteins, namely synucleins that are mainly expressed in brain and are localized to presynaptic terminals (8-10). Subsequent to the isolation of BCSG1, synuclein γ (6) and persyn (7) were independently cloned from a brain genomic library and a brain cDNA library. The sequences of these two brain proteins were found to be nearly identical to BCSG1. The five nucleotide difference found between BCSG1 and the sequences reported for synuclein γ and persyn are the results of natural nucleotide polymorphisms (7). Thus, BCSG1 is now also named synuclein γ or persyn and is considered to be the third member of the synuclein family.

*Neural protein synuclein*

Synucleins are a family of small proteins consisting of 3 known members, α synuclein (SNCA), β synuclein (SNCB), and γ synuclein (SNCG). The previously identified BCSG1 (also called SNCG), shares 54% and 56% amino acid **sequence identity** with SNCA and SNCB, respectively. The N-terminal halves of SNCA, SNCB, and SNCG are highly conserved. However, while the residues near the C-terminus of SNCA are similar to those of SNCB, those of SNCG diverge greatly from the SNCA counterpart (14). Although they are homologous, each synuclein is encoded by a different gene on chromosomes 4q21.3-q22 (SNCA), 5q35 (SNCB), and 10q23 (SNCG) (18). Synucleins are predominantly present in brain and thought to be involved in neuronal plasticity and the formation of depositions in brain tissues (19). In addition, synucleins are also present in ocular tissues, while α synuclein and β synuclein are predominantly present in the inner plexiform layer of retina, γ synuclein is in the fiber layer of optic nerve (20).

Synucleins has been specifically implicated in neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD). Mutations in SNCA is genetically linked to several independent familial cases of PD (21). More importantly, wild type of SNCA is the major component of Lewy bodies in sporadic PD and in a subtype of AD known as Lewy body variant AD (22-23). SNCA peptide known as non-amyloid component of plaques has been implicated in amyloidogenesis in AD (10,24). SNCB and SNCG have also been recognized to play a role in the pathogenesis of PD and Lewy bodies cases (8,25).

Although synucleins are highly expressed in neuronal cells and are abundant in presynaptic terminals, synucleins have also been implicated in non-neural diseases, particularly in the **hormone responsive** cancers of breast and ovary (1, 4-5, 11-17). Our data first indicate that the increased expression of SNCG correlates with breast progression and leads to more malignant metastatic phenotype (1, 11-12, 14). This is the first report indicating the potential involvement of synuclein in the non-neurotic disease. Others have also demonstrated the similar findings in ovary cancer (4-5, 16).

### I-2. Expression of SNCG in cancers

*Expression in breast cancer*

Being identified as a breast cancer specific gene, SNCG mRNA was detected in neoplastic breast epithelial cells but not in normal mammary epithelial cells (1). Northern blot analysis detected a 1 kb transcript corresponding to SNCG mRNA in 2/4 human breast cancer cell lines derived from pleural effusions and 4/4 breast cancer cell lines derived from ductal infiltrating carcinomas (1). *In situ*

hybridization analysis has demonstrated a stage-specific expression pattern of SNCG mRNA varying from virtually no detectable expression in normal or benign breast tissue to low level and partial expression in low grade breast carcinoma *in situ* to high expression in advanced infiltrating carcinomas (1). To confirm this stage-specific expression pattern of SNCG, we did RT-PCR analysis of SNCG expression in 36 clinical breast specimens including normal or benign lesions, stage II/III breast carcinomas, and stage IV breast carcinomas. While no BCSG1 mRNA was detectable in 7 breast specimens of normal or benign hyperplasia, BCSG1 mRNA was expressed in 43 % (6 of 14) and 73 % (11 of 15) of stage II/III and stage IV breast carcinomas, respectively (see **Fig. 1**). Western analysis to examine SNCG protein expression in human breast tissues showed a similar pattern in that it was not detected in normal breast tissues and stage I/II ductal breast carcinomas, but was detected in 70% of Stage III/IV ductal breast carcinomas (5). Ninkina et al were also able to confirm by using Northern and Western blotting that some breast tumors and breast tumor cell lines expressed SNCG, whereas normal breast tissue did not (6).

*Expression of SNCG in ovarian cancer.*

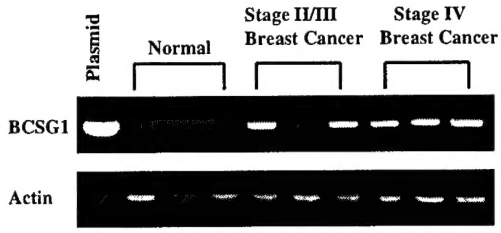
In addition to the link between SNCG and breast cancer progression, it has also been found that synucleins, especially γ and β synuclein, are involved in ovarian cancer. Following our identification of SNCG, Lavedan et al first suggested that SNCG may be abnormally expressed in ovarian tumors as well as in breast tumors, based on the discovery of some SNCG ESTs in the libraries derived from an ovarian tumor (6). This suggestion was further confirmed by Western and immunohistochemical analyses (5). While synucleins (α, β, and γ) expression was not detectable by immunohistochemistry in normal ovarian epithelium, 87% (39 of 45) of ovarian carcinomas were found to express at least 1 type of synuclein, and 42% (19 of 45) expressed all 3 synucleins (α, β, and γ) simultaneously. Highly punctate SNCG expression was also observed in 20% of preneoplastic lesions of the ovary, including epithelial inclusion cysts, hyperplastic epithelium, and papillary structures, suggesting that SNCG up-regulation may occur early in the development of some ovarian tumors (5).

**II. WORK ACCOMPLISHED.** The overall hypothesis to be evaluated is that SNCG play a critical role in breast cancer malignant progression leading to metastasis. The overexpression of BCSG1 may correlate with clinical aggressiveness of breast cancers. Therefore an alternations of BCSG1 expression may lead to an abnormal growth and malignant progression.

**Task 1: Biological relevance of BCSG1 expression to breast cancer progression**

**A. Screening of BCSG1 expression in clinical breast specimens.** FINISHED

Our in situ hybridization analysis has demonstrated a stage-specific expression pattern of BCSG1 mRNA varying from virtually no detectable expression in normal or benign breast tissue to low level and partial expression in low grade in situ breast carcinoma to high expression in advanced infiltrating carcinomas (1). In the grant proposal, we proposed to continue use in situ hybridization analysis of BCSG1 expression in clinical breast specimens. Because of the **non-quantitative** nature of in situ analysis, we performed a RT-PCR analysis in stead of the originally proposed in situ analysis. To verify the stage-specific expression pattern of BCSG1, we analyzed 36 clinical breast specimens including normal or benign lesions, stage II/III breast carcinomas, and stage IV breast carcinomas. As shown in Fig. 1, while no BCSG1 mRNA was detectable in 7 breast specimens of normal or benign hyperplasia, BCSG1 mRNA was expressed in 43 % (6 of 14) and 73 % (11 of 15) of stage II/III and stage IV breast carcinomas, respectively.




Expression of BCSG1/SNCG in human breast

| Stage | Normal (n=7) | Stage II/III (n=14) | Stage IV (n=15) |
|---|---|---|---|
| Expression | 0 (0%) | 6 (43%) | 11 (73%) |

**Fig. 1**. Expression of BCSG1 in human breast tissue. Total RNA was isolated from frozen human breast specimens. RT-PCR analysis of BCSG1 using primers within BCSG1 cording sequence (Sense: 5'-ATGGATGTCTTCAAGAAGGG-3'; antisense: 5'-CTAGTCTCCCCCACTCTGGG-3'). The 384-bp PCR product is a specific indication of the presence of BCSG1. The integrity and the loading control of the RNA samples were ascertained by actin expression with a set of primers (5'-GCTGTGCTATCCCTGTACGC-3' and 5'-TGCCTCAGGGCAGCGGAACC-3') for 314-bp β-actin

Using Western blot, Godwin AK's group also demonstrated a similar BCSG1 **protein** expression pattern in human breast samples. BCSG1 protein expression was not detectable in either normal breast or ductal carcinoma in situ (0 of 3) or Stage I/II breast carcinoma (0 of 6). However, 70% (12 of 17) of Stage III/IV breast carcinomas expressed SNCG protein. To emphasize the similarity and the importance of this stage-specific BCSG1 expression in breast tissue, PI downloaded Dr. Godwin's data here as Fig. 2 on BCSG1 expression in breast tumors.

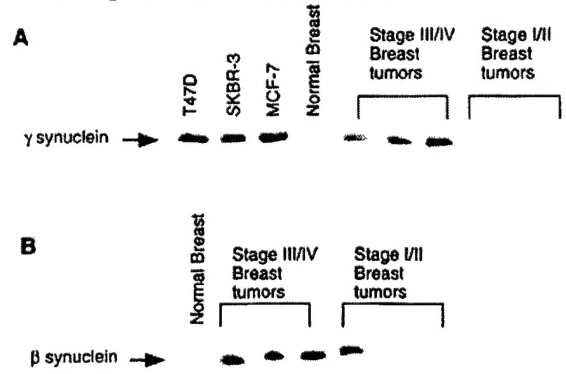



**Fig. 2**. Expression of γ and β synuclein in breast (Cancer 88: 2154-2163, 2000). Tissue extracts were prepared and screened by Western blotting. (A) the blot was probed with the anti-γ synuclein antibody. (B) the blot was probed with the anti-β synuclein antibody. Although β synuclein was expressed in some breast carcinomas of all stages, including ductal carcinoma in situ, SNCG expression was restricted to advanced Stage III/IV breast carcinomas; 82% (14 of 17) of the Stage III/IV breast carcinomas expressed either BCSG1, β synuclein, or both simultaneously.

**B. Effects of BCSG1/SNCG overexpression of on tumor growth and metastasis. FINISHED (11)**

This specific aim is finished and the data is published in Cancer Res. 59: 742-747, 1999 .

1. Overexpression of SNCG in breast cancer cells led to a profound augmentation metastasis *in vivo*.

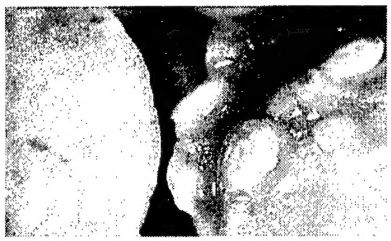


***Fig. 3*** *(from the Cancer Res Paper). Representative lung metastases from mice injected with SNCG transfected MDA-MB-435 clone and SNCG-negative neo-transfected control clone. Left lung was from the mouse injected with control MDA-MB-435 cells; right lung was from the mouse injected with SNCG transfected MDA-MB-435 cells.*

2. As to the mechanism(s) for the SNCG-induced invasion and metastasis, we demonstrated that SNCG-induced metastasis was not associated with the alternation in MMP and TIMP activity.
3. Mechanistically, the induced invasion and metastasis is related to SNCG-stimulated cell motility. We provided evidences linking overexpression of SNCG in human breast cancer cells with increased

motility and invasive activity *in vitro.* We also demonstrated that the SNCG-induced cell migration is independent of the serum gradient among the top and bottom chambers in the Boyden Chamber invasion assay. These data suggest that the increased migration in SNCG transfected cells is not likely to be mediated by chemotaxis but rather by cellular intrinsic high motile features.

**Task 2: Regulation of apoptosis by BCSG1/SNCG**

*SNCG contributes tumorigenesis by promoting tumor cell survival*

SNCG protects ovarian cancer cells from apoptosis and stimulates constitutive activation of ERK1/2 and down-regulation of JNK1. The effect of SNCG on apoptosis and activation of JNK and ERK in response to several chemotherapy drugs was investigated in ovarian cancer cells by Godwin AK's group (16). SNCG expressing cells are significantly more resistant to the chemotherapeutic drugs paclitaxel and vinblastine as compared with the parental cells. The resistance to paclitaxel can be partially obliterated when ERK activity is inhibited using a MEK1/2 inhibitor. Consistent with its anti-apoptotic effect, overexpression of SNCG leads to constitutive activation of ERK1/2 (extracellular signal-regulated protein kinase) and down-regulation of JNK1 (c-Jun N-terminal kinase) in response to a host of environmental stress signals including UV, arsenate, and heat shock. While the activation of ERK pathway leads to the cell survival, activation of JNK leads to the downstream caspase-3 activation and apoptosis. Taken together, these data indicate that oncogenic aberrant SNCG expression contributes to the development of breast and ovarian cancer by promoting tumor cell survival under adverse conditions and by providing resistance to certain chemotherapeutic drugs.

*Involvement of SNCG in the development of chemoresistance in colorectal cancer.*

In a search for novel targets that may lead to the development of chemoresistance of colorectal cancer cells, Lage H's group (15) has used two-dimensional electrophoresis to identify proteins that are overexpressed in colorectal cancer cells that are resistant towards mitoxantrone. Two target proteins have been identified by using mass spectrometry microsequencing and interestingly one of the target proteins is SNCG.

We have demonstrated a similar anti-apoptotic effect of SNCG on SNCG transfected MDA-MB-435 and MCF-7 cells. Currently we are investigating whether this anti-apoptotic effect is mediated by activation of JNK and ERK as reported in ovarian cancer cells (16).

**Task 3: Regulation of BCSG1 expression. FINISHED** (12)

This specific aim is finished and the data is published in Breast Cancer Research and Treatment 62: 99-107, 2000 .

1. Expression of BCSG1 mRNA in H3922 human breast cancer cells was significantly decreased by treating cells with the cytokine OM who has a growth-inhibitory effect on these cells. A decrease in BCSG1 mRNA level can be detected as early as 30 min after OM addition. By 4 h OM treatment, the level of BCSG1 mRNA was decreased to 70% of control, and by 24 h the mRNA was below detectable level (Fig. 1).
2. Western blot analysis further demonstrated the suppression of BCSG1 protein expression by OM. The level of SNCG protein in H3922 cells was reduced more than 90% by OM after 2 days (Fig. 3).
3. OM-induced down-regulation of BCSG1 mRNA occurred mainly at the transcriptional level (Fig. 4).
4. Examination of cell growth under anchorage-dependent and anchorage-independent conditions demonstrates that over expression of BCSG1 gene significantly stimulated the growth of MCF-7 cells both in monolayer culture and in soft agar. These data together suggest that BCSG1 may be one of the contributing factors that promote the uncontrolled growth of malignant **mammary** cells (Fig. 5).

**Additional new data.** What role SNCG has in breast and ovary and how it is implicated in breast and ovary cancer remains a mystery. The association between SNCG expression and the progression of

steroid dependent cancers of breast and ovary promoted us to investigate the role of SNCG in regulation of ER-α. A notable finding was that SNCG strongly stimulated the ligand-dependent transcriptional activity of ER-α. Augmentation of SNCG expression stimulated transcriptional activity of ER-α and estrogen-stimulated cell growth. The results demonstrated that human ER-α requires SNCG for efficient transcriptional activity.

*Overexpression of SNCG stimulated transcriptional activity of ER-α*

We measured the effect of SNCG on modulating the transcriptional activity of ER-α in MC-7 human breast cancer cells. MCF-7 cells were transiently transfected with either the pCI-SNCG expressing plasmid or control pCI-neo plasmid. Transfection of SNCG gene into the SNCG-negative MCF-7 cells did not affect ER-α expression (**Fig. 4A**) but significantly stimulated E2-mediated activation of ER-α (**Fig. 4B**). While treatment of wild-type MCF-7 cells with 17-β-estradiol (E2) activated estrogen-responsive reporter ERE4-Luciferase (ERE4-Luc), overexpression of SNCG gene in MCF-7 cells increased E2-stimulated reporter activity 3.2-fold over the SNCG-negative control cells. The SNCG-stimulated transcriptional activity of ER-α was ligand-dependent, because SNCG had no significant effect on the transcriptional activity of ER-α in the absence of E2.

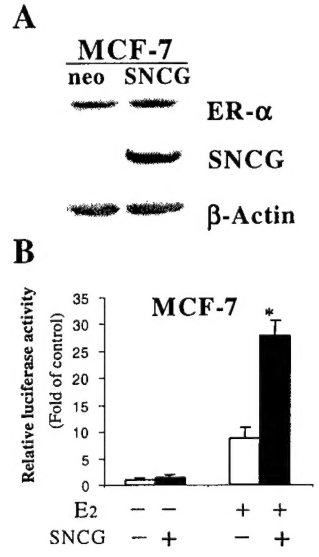



**Fig. 4**. SNCG stimulated ER-α transcriptional activity in MCF-7 human breast cancer cells. (**A**). Western analysis of ER-α and SNCG in MCF-7 cells transfected with pCI-SNCG or the control vector pCI-neo. Expression of SNCG did not affect the ER-α expression. (**B**). SNCG stimulated ER-α signaling MCF-7 cells. pERE4-Luc as well as control reporter pRL-SV40-Luc were co-transfected into SNCG-transfected and control neo-transfected cells. After transfection, cells were cultured in the ligand-free pheno-red free medium containing 5% stripped serum for 4 days, treated with or without 1 nM E2 for 24 hours before the promoter activities were determined by measuring the dual luciferase activity. The ERE reporter luciferase activity was normalized against the control renilla luciferase activity to correct for transfection efficiency. All values were presented as the fold induction over the control luciferase activity in the non-treated SNCG-negative cells, which was taken as 1. SNCG overexpression in ER-α-positive MCF-7 cells stimulated E2-activated reporter activity 3.2-fold over the SNCG-negative cells (*$p$<0.001).

*Stimulation of ER-regulated genes*.

Consistent with the increased transcriptional activity of ER-α, SNCG also stimulated E2-regulated gene transcription in MCF-7 cells (**Fig. 5**). While SNCG had no effect on the transcription of Cathepsin D, PS2, and TGF-α in the absence of E2, transcription of Cathepsin D, PS2, and TGF-α were increased 4.6-fold, 3.3-fold, and 4.2-fold in SNCG transfected cells vs. control cells in the presence of E2, respectively.

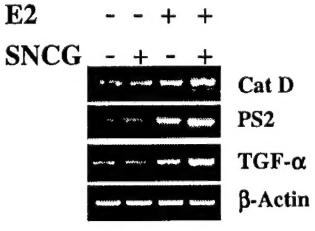



**Fig. 5**. SNCG stimulated estrogen-regulated gene transcription in MCF-7 cells. Cells were cultured in the ligand-free medium for 4 days. Cells were then treated with or without 1 nM of E2 for 8 hours before the isolation of mRNA. Expressions of mRNA of Cathepsin D (Cat-D), PS2, and TGF-α were studied in SNCG transiently transfected cells vs. control cells by RT-PCR analyses. A 842-bp product of Cat-D, a 336-bp product of PS2, and a 240-bp product of TGF-α, were amplified by RT-PCR.

*Stimulation of cell proliferation by SNCG*.

To determine the biological relevance of SNCG-stimulated ligand-dependent ER-α signaling, we analyzed the effect of SNCG overexpression on the growth of breast cancer cells. The cellular proliferation of the previously established two stable SNCG-transfected MCF-7 cell clones, SNCG-MCF-2 and SNCG-MCF-6, were compared with that of SNCG-negative cells, neo-MCF-1 and neo-MCF-2 (11). Data in **Fig 6** shows that while SNCG had no effect on the proliferation of SNCG-MCF cells compared to neo-MCF cells in the absence of E2, overexpression of SNCG significantly stimulated the ligand-dependent proliferation. Treatment of neo clones with E2 stimulated average cell proliferation 2.4-fold over controls. However, E2 treatment of SNCG clones resulted in an average of 5.4-fold increase in the proliferation vs. controls, suggesting that SNCG expression renders the cells more responsive to E2-stimulated cell proliferation. Consistent with its stimulatory effect on ligand-dependent cell proliferation, overexpression of SNCG did not affect the proliferation of ER-α-negative MDA-MB-435 cells (9).

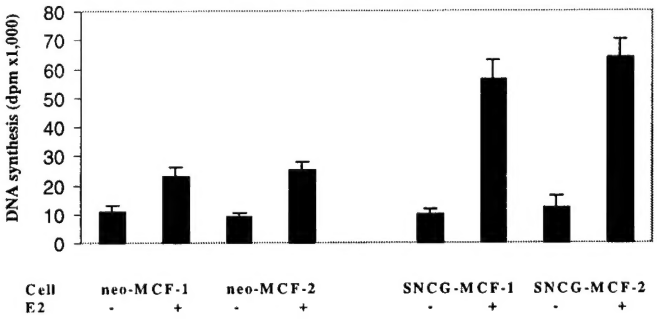



**Fig. 6**. SNCG stimulated ligand-dependent cell proliferation. Cells were cultured in the ligand-free Conditioned Cell Culture for 4 days and then treated with or without 10 nM E2 for 24 hours. Cell proliferation was measured by $^{3}H$- thymidine incorporation. Data are means ± SD of three cultures.

## III. KEY RESEARCH ACCOMPLISHMENTS:

1. Synuclein γ (SNCG), also referred as breast cancer specific gene 1 (BCSG1), is the third member of a neuronal protein family synuclein. SNCG is highly expressed in human infiltrating breast carcinomas but not expressed in normal or benign breast tissues.
2. Overall SNCG mRNA expression was detectable in 38% breast cancers. However, 79% of stage III/IV breast cancers were positive for SNCG expression, while only 15% of stage I/II breast cancers were positive for SNCG expression. This study suggests that the expression of SNCG is stage-specific for breast cancer. SNCG is expected to be a useful marker for breast cancer progression and a potential target for breast cancer treatment.
3. SNCG expression protects cells from apoptosis. This anti-apoptotic effect may be mediated by activation of ERK1/2 and down-regulates JNK1.
4. One of the targets for aberrant SNCG expression in breast cancer is to regulate ER-α transcriptional activity. Overexpression of SNCG significantly stimulates ER-α transcriptional activity.

## IV. REPORTABLE OUTCOMES AND CONCLUSIONS:

1. We have recently identified and cloned a putative breast cancer specific gene, BCSG1, also named as SNCG. We have demonstrated that expression of SNCG correlate with clinical aggressiveness and may indicate breast cancer malignant progression leading to metastasis. Expression of SNCG in breast carcinoma is stage specific: while SNCG expression is not detectable in normal or benign lesions and lower percentage of SNCG is detected in Stage I/II breast carcinomas, but up to 70% of stage III/IV breast carcinomas express SNCG. This stage-specific SNCG expression in breast tissue has been demonstrated, so far, in three assays: in situ hybridization analysis of 53 clinical specimens (1), RT-PCR analysis of 37 clinical specimens (manuscript in preparation), and Western analysis of 26 clinical specimens (5).
2. We also provided evidences linking overexpression of SNCG in human breast cancer cells with increased migratory motility and invasive activity *in vitro* and a profound augmentation of metastasis *in vivo*. These data suggest that expression of SNCG correlates with breast cancer progression.

Therefore, analysis of SNCG expression may be useful in staging breast carcinomas, or predicting clinical outcomes; for example, a women whose breast tumor tests positive for SNCG expression is likely to have a more aggressive and invasive tumor than a woman whose breast tumor does not express SNCG. Thus, the SNCG positive woman's disease should perhaps be treated more aggressively. The notion that the SNCG overexpression may indicate and facilitate breast cancer malignant progression from a benign breast or *in situ* carcinoma to a highly infiltrating carcinoma warrants further investigation.

3. Although we have demonstrated that aberrant expression of SNCG correlates nicely with breast cancer development and progression, the molecular targets of SNCG aberrant expression for breast cancer have not been identified. Here we demonstrated ER-α as one of the critical target molecules for SNCG's action in breast cancer pathogenesis. Our findings suggest that SNCG functions as a chaperone for efficient activation of steroid receptors. Thus, aberrant expression of SNCG stimulates breast cancer growth and progression, at least in part, by enhancing the transcriptional activity of ER-α.

## V. TRAINING

This is PI's first independent grant. The proposed studies of the current grant application includes a variety of different aims and experiments ranging from basic molecular biology, cell biology, *in vivo* orthotopic nude mice model for tumor growth and metastasis, and a clinical oriented study on screening clinical human breast specimens. This is the first time that PI has a chance to independently carry out a very challenge, yet ambitious, multi display project. During the last year, PI has gained a lot of experience on animal model and *in vivo* analysis of tumor metastasis. The success on the current grant proposal will encourage and facilitate PI's future career development as an independent clinically oriented breast cancer investigator. Currently, PI is intended to develop a BCSG1 transgenic mice model under the control of mammary specific promoter MMTV. This transgenic model will be used to evaluated 1) the effect of BCSG1 overexpression on mammary development and induction of mammary tumor in MMTV/BCSG1 transgenic mice; and 2) the role of overexpression of BCSG1 in breast cancer progression in MMTV/BCSG1 transgenic mice. Hopefully, with the successful development of MMTV/BCSG1 transgenic mice model, PI will be ready for a RO1 grant submission.

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